Cross-Fertilization for Enhancing Tocotrienol Biosynthesis in Rice Plants and QTL Analysis of Their F_2 Progenies

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As rice bran tocotrienol (T3) has been known to have a wide range of physiological functions (e.g., antiangiogenesis), we aimed at developing a T3-rich rice variety for nutraceutical purposes. T3 content in more than 250 kinds of rice bran samples were investigated, and Milyang23 was found as the best variety rich in T3. The variety was therefore chosen for cross-fertilization with Koshihikari. Among obtained F_2 progenies, some of them became improved in T3 content (up to 2-fold of reference Koshihikari). QTL analysis of the F_2 progenies revealed five putative loci corresponding to T3 biosynthesis, in which the main loci were located near a marker RM3827 on chromosome 6. The results show that cross-breeding is effective in improving rice bran T3 and provides more genetic understanding on T3 biosynthesis in rice plants.

INTRODUCTION

Rice bran, the combined part of pericarp, seed coat, nucellus, and aleurone layer or rice seeds, has been known to contain functional compounds such as tocotrienol (T3) and tocopherol (Toc). Among these compounds, T3, an unsaturated form of vitamin E with three double bonds in its isoprenoid side chain (**Figure 1**), has recently been receiving considerable attention for its several biological properties (*1*). T3 shows better antioxidative (*2*), antihypercholesterolemic (*3*), anticancer (*4*), and neuroprotective activities (*5*) than Toc. In addition, we have found that T3 suppresses pathological angiogenesis (*6−8*), which is the important stage in the progression of some disorders (i.e., diabetic retinopathy, rheumatoid arthritis, and cancers). These findings suggest that T3 has a wide range of physiological functions, and developing a rice variety that can biosynthesize high amounts of T3 would be useful for nutraceutical applications.

Considering the biosynthesis of T3 in plants (e.g., rice and barley), T3 is synthesized together with Toc in plastids from precursors derived from the shikimate and methylerythritol phosphate pathways (*9, 10*), and homogentisic acid geranylgeranyl transferase (HGGT, belonging to plant prenyltransferases) has been believed to work as the key enzyme for regulating T3 production. In support of this, it was reported that transgenic expression of the barley HGGT in *Arabidopsis thaliana* leaves resulted in the accumulation of T3, which was absent in its nontransgenic leaves (*11*). Therefore, biosynthesis of T3 in plants would be genetically controlled, but the genetic regulation of activity of HGGT as well as other enzymes for T3 production in rice has been poorly understood. In our previous study, we found a wide variation of T3 content among several rice bran samples (*12*), which may be an outcome of their difference in genetic characteristics for T3 production. The findings hypothesize that cross-breeding would be effective in improving T3 content in rice cultivars by genetically increasing the activity of the enzymes (e.g., HGGT) for T3 biosynthesis.

Accordingly, as we aim at developing a rice variety that can synthesize a high level of T3, a series of studies was conducted. A number of rice bran samples were determined for their T3 and Toc contents by using our previously developed method (*12*), and the best rice cultivar that was able to produce a reliable high content of T3 was then chosen. The chosen variety was crossed with *japonica* Koshihikari, the most popular rice variety in Japan, resulting in a wide distribution of T3 content in their F_2 progenies. Some of the F_2 individuals showed markedly high T3 levels, and the F_2 progenies were useful in evaluating the T3 biosynthesis in rice plants by using a quantitative trait locus (QTL) analysis.

MATERIALS AND METHODS

Chemicals. Four isomers of T3 ($α$-, $β$-, $γ$-, and $δ$-T3) were gifts from Eisai (Tokyo, Japan). Four isomers of Toc ($α$-, $β$-, $γ$-, and $δ$-Toc) and 2-propanol were obtained from Wako (Osaka, Japan). All reagents used were of analytical grade.

Rice Bran Samples and Vitamin E Analysis. The seeds of more than 250 kinds of rice varieties including the rice diversity research set of germplasm (RDRS) provided by National Institute of Agrobiological

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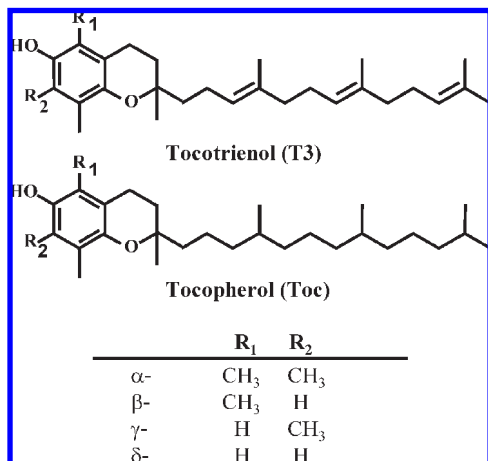
Figure 1. Chemical structure of tocotrienol (T3). T3 has an unsaturated isoprenoid tail, which differs from tocopherol (Toc) bearing a saturated phytyl side chain.
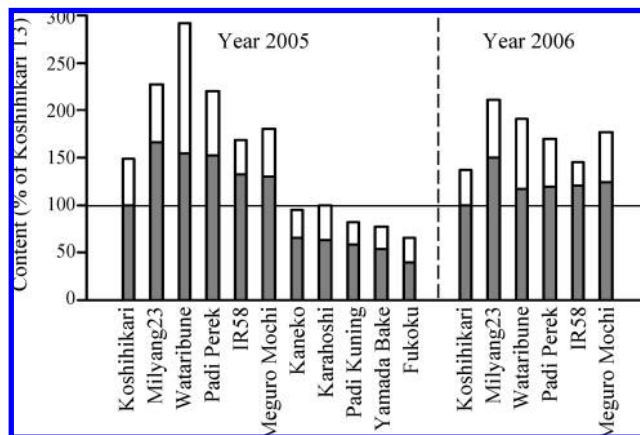

Figure 2. Total T3 (shown in gray) and Toc (shown in white) content of some rice varieties. The reference Koshihikari T3 was expressed as 100%.

Figure 3. Frequency distribution of T3 content of the 133 F_2 individuals of Koshihikari and Milyang23. Values are expressed as % T3 compared with that of the parental Koshihikari.

Sciences (NIAS) Genebank were surveyed. Twenty seedlings of each variety were individually transplanted by 25×25 cm^2 spacing at the experiment farm of Toyama Prefectural Agricultural, Forestry and Fisheries Research Center (Toyama, Japan) in 2005. The whole rice crops were dehulled, and rice bran samples were prepared (*12*). The rice bran samples were stored at -30 °C in a humidity-controlled environment until analysis. The extraction of T3 and Toc from the rice bran samples was done by using one-step equilibrium direct solvent extraction using 2-propanol, and the quantitation of T3 and Toc was employed using normal phase high-performance liquid chromatography with a fluorescence detector (HPLC-FL) as described previously (*12*). Similarly, seedlings were transplanted in 2006, and rice bran samples were determined for their T3 and Toc by HPLC-FL for investigating the yearly change of vitamin E content.

Cross-Fertilization. After a number of rice bran samples (prepared in 2005 and 2006) were determined for their T3 and Toc content, a T3-rich variety (*indica* Milyang23) was selected and cross-fertilized with *japonica* Koshihikari. Briefly, pollen emasculation was applied by soaking panicles of seed parents in hot water at 42 °C for 7 min just before flowering. Hybridization was done in very humid conditions because ovules could be easily damaged by drying (*13*). F_2 seed populations of Milyang23 and Koshihikari were raised after F_1 self-fertilization. T3 and Toc contents in the rice bran of the F_2 individuals were determined by HPLC-FL. The samples of F_2 individuals and the parental varieties were also subjected to genetic analysis as described later.

Simple Sequence Repeat (SSR) Marker Analysis. Total DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method (*14*) from leaves of the F_2 individuals and the parental lines. Polymerase chain reaction (PCR) was performed in a 10-μL reaction mixture containing 0.25 units of *G-Taq* DNA polymerase (Hokkaido System Science, Hokkaido, Japan), 0.35 mM of dNTPs, 20 ng of DNA, and 5 pmol of each primer. The PCR amplification for SSR was performed as follows: 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s, using DNA Engine TETRAD2 (Bio-Rad, USA). To detect parental polymorphism, the PCR products were electrophoresed on a 3% agarose gel at 200 V for 100 min, each with 0.5× Tris-borate and EDTA buffer. The PCR products were stained with ethidium bromide and photographed under UV light. We screened 252 SSR markers distributed across the entire 12 chromosomes for parental polymorphism and used the SSR markers that showed polymorphism for the analysis of the F_2 progenies.

Linkage Analysis. Linkage analysis was performed with MAP-MAKER/EXP 3.0 (*15*). We used the 'ri self' mode to determine the linkage groups and marker orders. To determine the linkage between two markers, a level of the odds ratio (LOD) threshold of 2.5 was used. The map distance was obtained by using the Kosambi mapping function (*16*). Chromosome assignment was based on the map location of the SSR markers corresponding to the chromosomes determined in a previous linkage map of rice (*17*).

QTL Analysis. Genotype data of SSR markers that showed polymorphism were used for QTL analysis. Chromosomal locations of
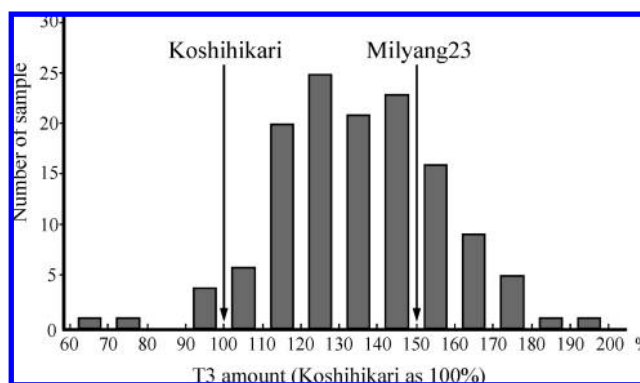
putative QTLs were determined by composite interval mapping by using Cartographer v.2.5 (*18*). The experiment-wise logarithm of the significant LOD threshold was determined by computing 1000 permutations of the T3 contents in QTL Cartographer. The detection threshold for QTLs in this study was LOD = 2.50, which approximately corresponded to experiment-wise P values of 0.05.

RESULTS

Variation of T3 Contents in Different Varieties of Rice Bran. More than 250 samples of rice bran (prepared in 2005) were determined for their T3 and Toc. The quantitative data of T3 and Toc content of some varieties (Koshihikari, first 5 and last 5 quantitatively ordered varieties) are reported in **Figure 2**. As a result, a large variation of vitamin E contents was observed in rice bran samples. Among vitamin E compounds, γ-T3 and β-T3 were the predominant and the smallest constituents, respectively. In case of the reference Koshihikari, its T3 concentration was 880 μg/g (290, 560, and 30 μg/g for α-, γ-, and δ-T3, respectively). The variety containing the highest level of T3 was Milyang23 (166% T3 to the reference), and the variety having the lowest content of T3 was Fukoku (40%). The quantitative data of the rice bran samples prepared in 2006 also revealed that Milyang23 was the best variety having a high content of T3 (**Figure 2**), and Milyang23 was therefore chosen for cross-fertilization with Koshihikari.

T3 Contents in F_2 of Koshihikari and Milyang23. After the cross-fertilization, 133 F_2 individuals of Koshihikari and Milyang23 were obtained. We found a frequency distribution of T3 content (total of T3 isomers) of the F_2 rice bran samples that ranged from 60 to 200% (average content was 135%) compared to that of rice bran from parent Koshihikari (**Figure 3**). From a total of
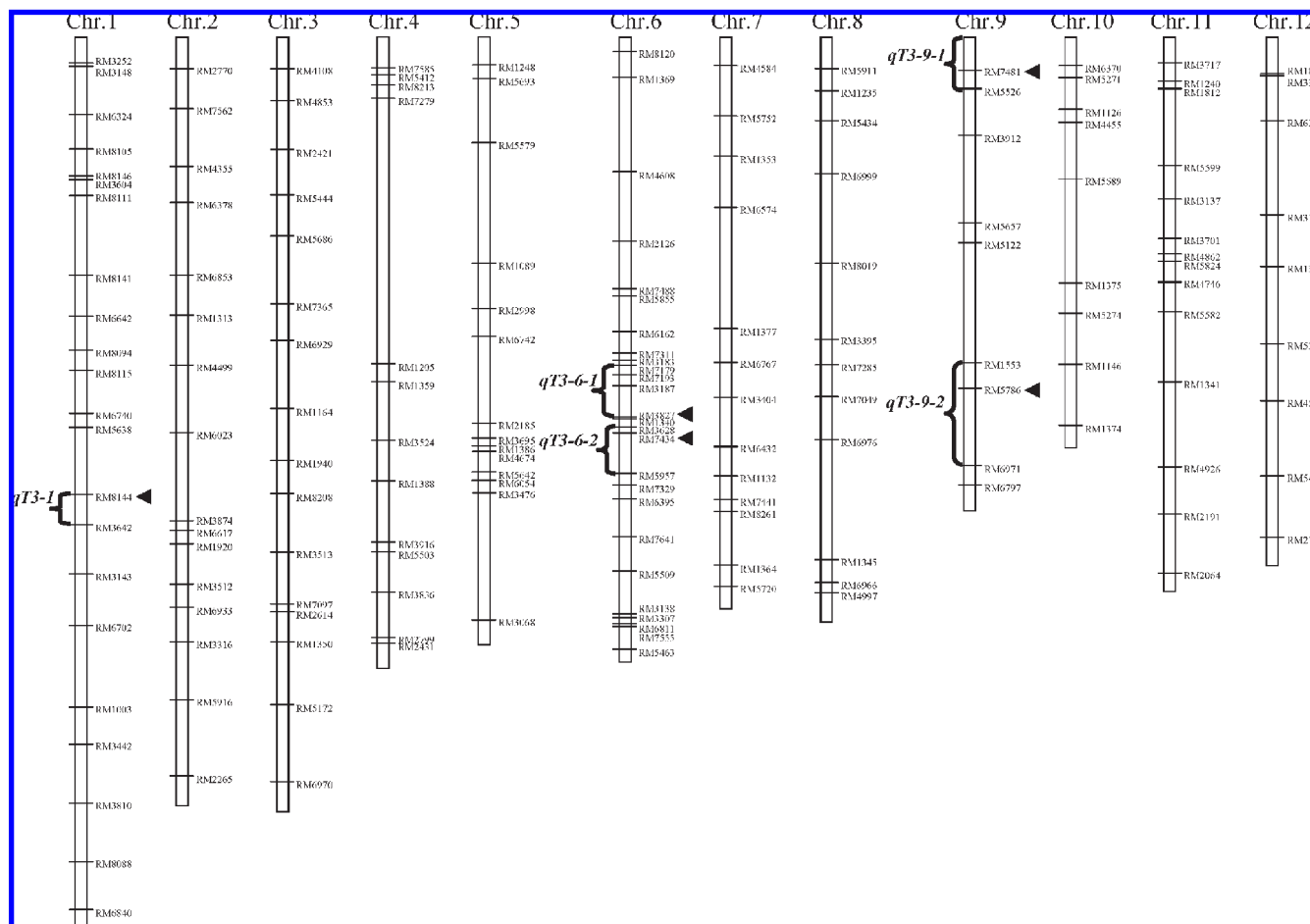
Figure 4. A genetic linkage map of 173 SSR markers and the chromosomal positions of putative QTLs for the high T3 production of rice. SSR markers are listed on the right side of chromosomes. Arrowheads denote the nearest markers to each putative QTL.

Table 1. Information for Each of the Putative QTL for High T3 Production

QTL	NML[a]	chromosome	LOD	AE[b] (μg/g)
qT3-1	RM8144	1	2.7	−69.4
qT3-6-1	RM3827	6	11.3	−23.6
qT3-6-2	RM7434	6	10.2	−25.8
qT3-9-1	RM7481	9	3.9	30.7
qT3-9-2	RM5786	9	4.2	−56.2

[a] NML: nearest marker locus to putative QTL. [b] AE: additive effects of the Koshihikari allele.

133 samples of the F_2, there were 6 samples with T3 content less than that of Koshihikari (5% of the total populations), 95 samples with T3 content between that of Koshihikari and that of Miyang23 (71% of the total populations), and 32 samples with T3 content more than that of Milyang23 (24% of the total populations). Of the 32 samples, the highest level was 1740 μg/g (225, 25, 1460, and 30 μg/g for α-, β-, γ-, and δ-T3, respectively). These results indicated that cross-breeding was useful in improving T3 content in rice cultivars.

QTL Analysis for the Incidence of High T3 Biosynthesis. In this study, a genetic linkage map of the 173 SSR markers showing polymorphism between the parental Koshihikari and Milyang23 was constructed and used (**Figure 4**). Composite interval mapping was employed to identify the QTLs corresponding to the incidence of T3 biosynthesis of the rice variety, on the basis of the 133 F_2 individuals of Koshihikari and Milyang23. Five putative QTLs associated with the T3 biosynthesis were detected on chromosome 1 (*qT3-1*), chromosome 6 (*qT3-6-1* and *qT3-6-2*),

and chromosome 9 (*qT3-9-1* and *qT3-9-2*) (**Figure 4**). The chromosomal positions and additive effects of these QTLs are summarized in **Table 1**. To the detected QTLs, most of them were from genomic characteristics of Milyang23 (*qT3-1*, *qT3-6-1*, *qT3-6-2*, and *qT3-9-2*), while only single QTL, were from those of Koshihikari (*qT3-9-1*). Of these QTLs, we found that the *qT3-6-1* with the highest LOD score of 11.3 for T3 biosynthesis was located on chromosome 6 and was mapped near the SSR marker RM3827 (**Figure 5**).

DISCUSSION

As increasing evidence supports the biological role of T3 (*1−8*), researchers have been focusing on the biofortification of T3 (*19*). Up to date, T3 enhancement in some plants has been done, for example, Cahoon et al. have reported that T3 could be accumulated up to 260 μg/g corn seeds (*11*). However, despite rice being regarded as one of the most important crops in the world, its biofortification of T3 has never been attempted. We previously found a clue that there was a wide variation of T3 content among rice bran samples possibly due to their difference in genetic characteristics for T3 production (*12*), and we therefore conducted this cross-breeding study for improving T3 content in rice cultivars.

On the basis of quantitative data of T3 in rice bran from different years (2005 and 2006) (**Figure 2**), we chose Koshihikari as one of cross-parents by considering the following reasons: (1) Koshihikari is one of the most highly grown varieties of rice in Japan partly due to its excellent eating quality; (2) despite these advantages of Koshihikari, its T3 content was not so high in

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comparison with that of other determined rice bran samples. For another cross-parent, Milyang23 was chosen because Milyang23 was the variety rich in T3 content with less yearly change compared with that of other cultivars (**Figure 2**). Milyang23 is a
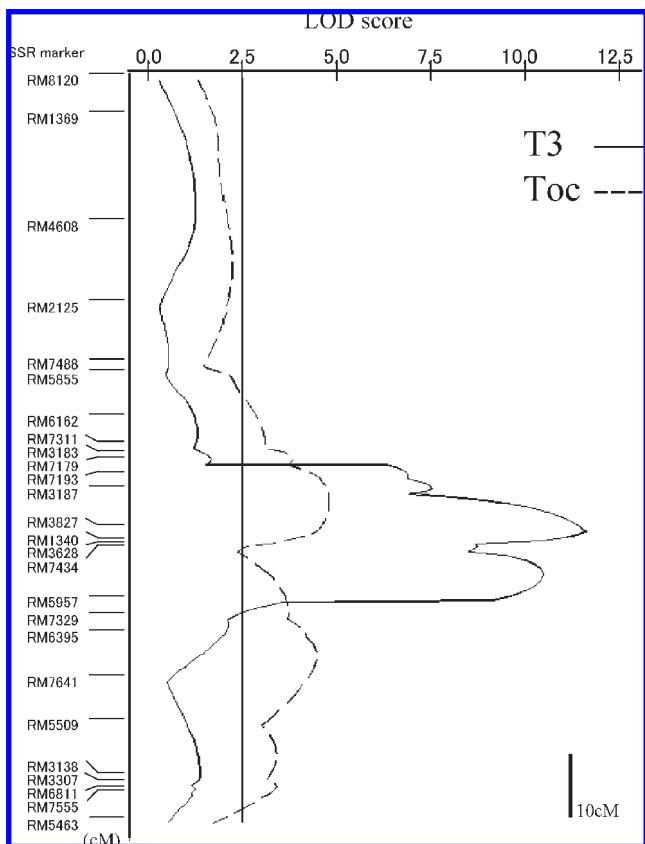

Figure 5. High resolution of LOD score plot on chromosome 6 for the QTLs for the occurrence of high T3 production in the F_2 populations of Koshihikari and Milyang23. The detection threshold was LOD = 2.50.

genetically divergent tongil-type rice (a high yielding plant type derived from an *indica* × *japonica* cross in Korea and similar to *indica* in its genetic makeup) (*20*). Our research team has long been using Milyang23 as a parent in breeding programs at National Agricultural Research Center for Tohoku Region and at Agricultural Experiment Station, Toyama Agricultural Research Center. Therefore, these experiences were helpful for developing T3-rich rice bran by cross-breeding of Koshihikari and Milyang23. On the other hand, genetic engineering has been proven to enhance vitamin E production in plants (*11*), and to directly regulate rice enzymes (i.e., HGGT), T3 content in rice seeds would have to be improved. However, because there are many issues for the potential hazard of genetically modified plants (*21, 22*), in this study a cross-breeding method was selected and conducted.

Recent understanding of plant metabolism has made it possible to increase micronutrient content in staple foods by plant cultivation (*23*). Among cultivation methods, cross-breeding is the process with the intention of creating progenies that share the traits of their parents. This suggested that when Koshihikari was crossed with Milyang23, some of their progenies were expected to be rich in T3 content and have inherited advantages of their parents Koshihikari and Milyang23 such as excellent eating quality and high yield properties. As shown in **Figure 3**, although there were only 6 F_2 individuals with T3 content less than that of the parent Koshihikari, the cross between Koshihikari and Milyang23 caused an overall improvement in T3 contents of their progenies in which the highest T3 content was 198% to Koshihikari T3 (132% to Milyang23 T3). The results support that cross-breeding is one of the effective tools for improving the nutrition of rice, which is in agreement with other studies considering an enhancement of protein and trace mineral in rice (*24, 25*). We speculated that the difference in the distribution of T3 levels in the F_2 (**Figure 3**) would be a result of the variation of genetic traits of both parental lineages.

To evaluate this speculation, we conducted a QTL analysis. The genetic linkage map was constructed using 173 SSR markers, which showed polymorphism between the parental Koshihikari
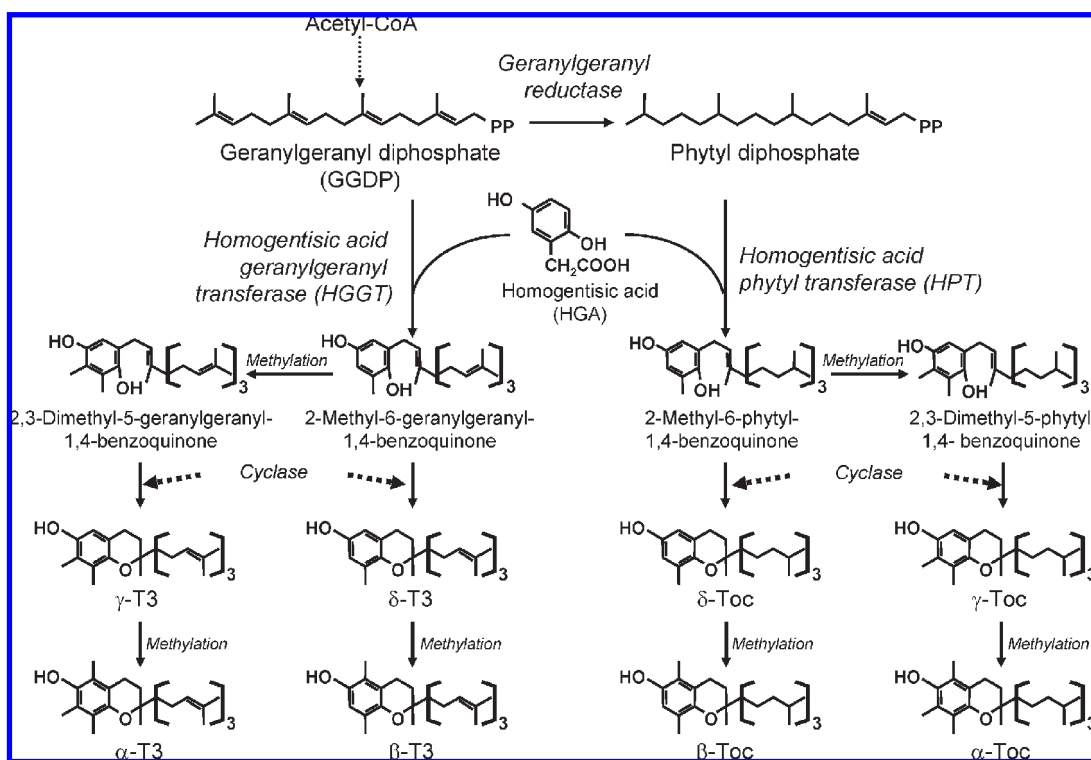
Figure 6. Pathways for the biosynthesis of T3 and Toc (*26*).

and Milyang23, and five putative QTLs for T3 biosynthesis were discovered (**Figure 4**). Of the five QTLs, the major QTL (*qT3-6-1*, LOD = 11.3; **Table 1**) was located near the marker RM3827 on chromosome 6 (**Figure 5**). Also, we found QTLs for Toc synthesis using the F_2 population; the QTLs were on chromosome 1 (*qTOC-1-1*; LOD = 2.5, near marker RM8094 and *qTOC-1-2*; LOD = 3.2 near marker RM3642), chromosome 3 (*qTOC-3*; LOD = 2.5 near marker RM5172), chromosome 6 (*qTOC-6-1*; LOD = 4.7 near marker RM3187 and *qTOC-6-2*; LOD = 4.4 near marker RM6395), chromosome 7 (*qTOC-7*; LOD = 2.7 near marker RM1132), and chromosome 8 (*qTOC-8*; LOD = 2.6 near marker RM6999), in which QTLs with the highest LOD score (*qTOC-6-1* and *qTOC-6-2*) were also located on chromosome 6. According to a recent understanding of the biosynthesis of plant vitamin E (*26*) (**Figure 6**), HGGT and homogentisic acid phytyl transferase (HPT) are believed to be the key enzymes for regulating of T3 and Toc production. As close chromosomal positions of *qT3-6-1*, *qTOC-6-1*, and *qTOC-6-2*, QTLs on chromosome 6 would have close relationship to T3 as well as Toc biosynthesis, suggesting that HGGT and HPT would have partial structural similarity as reported in a previous study (*27*). Therefore, for other QTLs located outside chromosome 6, they may not directly affect HGGT and HPT but may enhance other enzymes related to vitamin E precursors (i.e., acetyl CoA, geranylgeranyl diphosphate (GGDP), and homogentisic acid (HGA)) or even supplementary enzymes supporting the activity of HGGT and HPT. In our results, the improvement of T3 in the F_2 was mainly from the increased amount of γ-T3 (with little changes in amounts of α-T3 and δ-T3), hypothesizing that T3-rich F_2 may be enhanced for their production of 2,3-dimethyl-5-geranylgeranyl-1,4-benzoquinone (an intermediate of γ-T3 synthesis) in the synthetic process of T3 (**Figure 6**). On the other hand, in our results (**Figure 3**), there were 6 F_2 individuals with T3 content less than that of parent Koshihikari and 32 individuals with T3 content higher than that of parent Milyang23. This could be explained by the fact that the F_2 progenies low (or high) in T3 inherited inferior (or superior) QTL regions for T3 production from both parents.

In conclusion, we screened rice bran samples and found Milyang23 as the T3-rich rice variety. Koshihikari was then crossed with Milyang23, resulting in improved T3 amounts of their progenies (F_2). QTL analysis revealed five putative QTLs corresponding to T3 biosynthesis, which provide new insights into how to genetically regulate the activity of enzymes for T3 production in rice.

ABBREVIATIONS USED

GGDP, geranylgeranyl diphosphate; HGA, homogentisic acid; HGGT, homogentisic acid geranylgeranyl transferase; HPLC-FL, high-performance liquid chromatography with a fluorescence detector; HPT, homogentisic acid phytyl transferase; PCR, polymerase chain reaction; QTL, quantitative trait locus; SSR, simple sequence repeat; Toc, tocopherol; T3, tocotrienol.

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